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YEAST CELL WALL PEPTIDES AND ANTIBODIES THERETO

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Provisional Applications 60/120,764, 60/120,765, and 60/122,216, the contents of which are incorporated herein by reference

GOVERNMENT RIGHTS

This invention was made in part with United States Government support under Grants R29 AI31048, R01 AI3048, and F32 AI09428, awarded by the United States Public Health Service / NIH. The United States Government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to yeast cell wall proteins, monoclonal antibodies that recognize them, and methods for the detecting pathogenic yeast cells and treating disease associated with such organisms.

BACKGROUND OF THE INVENTION

Various yeasts are pathogens to humans. Cell surface hydrophobicity is involved in the adhesive properties and pathogenesis of many yeast strains, particularly the most common yeast pathogen, *Candida albicans*. Cell filaments, including hyphae and pseudohyphae, produced by these organisms during disease are hydrophobic. Hydrophobic cells bind more readily than hydrophilic cells to epithelial cells and plastics, and display more generalized binding to host tissues. Additionally, hydrophobic cells demonstrate greater resistance to phagocytic attack than do hydrophilic cells, and demonstrate greater virulence in mice.

Cell wall proteins are thought to contribute to, if not determine, the cell surface hydrophobicity of yeast cells. It has been shown that hydrophobic proteins are present on the cell wall surface of numerous

yeasts *in vitro*. The adhesion of these microorganisms to host tissue is an initial step in the colonization of new environments and is thought to play an important role in the pathogenesis of yeast cells.

Hydrophobic Characteristics of Fungal Cell Walls

Yeasts and molds can expose a cell surface that is hydrophobic despite living in an aqueous environment. The presence of a hydrophobic surface on a fungal cell provides a mechanism of attachment to substrata and, for molds, aerial hyphal formation. How yeasts and molds make a hydrophobic surface in an aqueous environment appears to differ. Molds produce small hydrophobic proteins. The proteins are generally classed as hydrophobins, a group of proteins that share three characteristics: size; eight cysteine residues in a conserved pattern; and hydrophobic domains arranged in a similar pattern. Surface hydrophobicity of the yeast-form *Candida* species appears to be due also to proteins. However, the proteins do not conform to hydrophobins. The proteins are present on vegetative cells and are larger in mass than hydrophobins. They do not appear to align as filamentous layers on the cell surface but may be associated with radiating fibrils that are mannosylated. At least one hydrophobic protein is shared by multiple species of *Candida* and by *Saccharomyces cerevisiae*. *Candida albicans* also contains a unique hydrophobic protein. Recent partial protein sequence analysis of the two hydrophobic proteins of *Candida albicans* suggests that the proteins share some sequence.

Introduction

Fungal cell surfaces can be hydrophobic. How an organism makes a hydrophobic surface and what the architectural arrangement of the hydrophobic surface differs depending on the form of the organism and cell structure that is involved. For example, it is not surprising to find that

vegetative cells, that is, cells undergoing continual cell growth, differ in their hydrophobic surface from resting cells or spores. Molds and yeasts appear to differ in what molecules determine surface hydrophobicity.

Hydrophobins

Molds produce proteins that confer surface hydrophobicity. The proteins, designated hydrophobins, are typically found on the surface of conidia and fruiting body tissues (e.g., ascostroma) (Wessels, J.G.H. (1996) *Trends in Plant Science* 1,9-15). They may also be the responsible molecules of certain fungal toxins, such as cerato-ulmin toxin). All hydrophobins share certain characteristics:

1. They have eight cysteine residues, which are generally arranged in a similar pattern along the protein;
2. They have long stretches of hydrophobic amino acids (10-20 aa);
3. They have hydrophobic regions arranged in a similar pattern;
4. They are small in mass (<20 kDa);
5. They have a conserved intron site;
6. They are produced only when the mycelium has reached a certain state of maturity.

Hydrophobins are secreted into the medium and form polymers on the hyphal surface (Wessels, J.G.H. (1996) *Trends in Plant Science*, 1, 9-15; Wösten, H.A.B. (1993) *Plant Cell* 5, 1567-1574; Wösten, H.A.B. (1994) *EMBO J.* 13, 5848-5854). These polymers can lead to aerial hyphal formation. Aerial hyphae that secrete hydrophobins will secrete the hydrophobins as insoluble complexes.

Given that hydrophobin genes are seen in the perfect subdivisions, it would seem likely that the surface hydrophobic proteins of the deuteromycetous yeasts would be related to the hydrophobins. However, two teleologic arguments for why the proteins should be different are:

1) Yeasts are vegetative cells. To secrete highly hydrophobic proteins that then polymerize on the surface of the cells would essentially lock the cell in stasis, i.e., not allow growth expansion.

2) Yeasts may reach sites with high nutrient value, but they do this within an aqueous environment. To release hydrophobic molecules to become aerobuoyant or aquabuoyant would result in poor distribution within an aqueous environment. A mixed population of hydrophobic and hydrophilic surface molecules could enhance the ability of the organism to attach to nutritionally rich sites.

Hydrophobic surface molecules of yeasts

Investigations into the hydrophobic molecules of *C. albicans*, perhaps the best studied yeast, suggest that the hydrophobic molecules differ from the hydrophobins of molds. For example, the hydrophobic molecules of *C. albicans* are:

1. Higher in mass (>20 kDa).
2. Produced constitutively.
3. Do not appear to have long stretches of hydrophobic amino acids.
4. Probably have short sequences of hydrophobic amino acids.

The production of hydrophobic wall proteins occurs during all growth phases and during yeast and hyphal morphogenesis of *C. albicans*. The proteins appear to be the same regardless of growth phase (Hazen B W, and Hazen K C. (1998) *Infect Immun* 56, 2521-2525; Hazen K C, and Hazen B. (1993) *FEMS Microbiol Lett* 107, 83-88). The proteins have been detected on early buds, late buds, and mature yeast cells, suggesting the proteins are made during all cell cycle phases.

We have focused on three surface hydrophobic proteins, which we designate CAgp40, CAgp38, and CAgp37 and for which we have monoclonal antibodies (Masuoka, J. et al. (1999) *FEMS Immunol. Med.*

Microbiol. 24:421-429). These proteins are larger in mass compared to the hydrophobins.

A protein with the same mass as CAgp40 and cross-reactive epitopes to CAgp40 was present in all *Candida* species we tested (seven species) and in *Saccharomyces cerevisiae*. It is possible that only the epitopes are shared among the species, but it is surprising to find in this case that the epitope-bearing protein was also the same mass in all the *Candida* species.

The protein CAgp38 was found only with *C. albicans*, although *C. tropicalis, S. cerevisiae,* and *C. kefyr* had proteins with cross-reactive epitopes but the epitope-bearing proteins were different in mass (Table 1).

Table 1. Detection by western blot of major cross-reactive protein bands from various yeast species by MAbs directed against *C. albicans* hydrophobic proteins.

	Mass and staining intensity of proteins detected by monoclonal antibody		
Yeast Species	**5F8-E10**	**6C5-H4**	**5D8-A12**
C. albicans	40	38	37
C. tropicalis	40	39, 54-55	37, 40
C. glabrata	40,55-56	--	--
C. gluilliermondii	40	--	--
C. parapsilosis	40	--	37
C. kefyr	40, 55, 59	36	--
C. krusei	40	50	--
S. cerevisiae	40, 45, 54	55 (3+)	35, 39
B. dermatitidis yeast	65,130	--	--

The protein CAgp37 epitope bearing proteins were present in wall extracts from several *Candida* species in the form of a 37 kDa protein *S. cerevisiae* also had cross-reactive epitope bearing proteins but their mass was different from the proteins from the *Candida* species.

When CAgp40 and CAgp38 were analyzed for amino acid sequence by mass spectrometry, we obtained at least nine peptides from each which were at least partially sequenceable. No extensive lengths of contiguous hydrophobic amino acids were obtained, but at least one peptide had a sequence that conformed to the rules for surface exposure of hydrophobic groups on a protein (3–5 hydrophobic amino acids surrounded by polar amino acids). In addition, the two proteins share a tripeptide sequence. The tripeptide may be a marker for such proteins.

Role of mannosylation in surface exposure of hydrophobicity by yeasts

C. albicans is unusual compared to other *Candida* species in that its surface hydrophobicity when grown to stationary phase varies depending on growth temperature. In addition, its surface hydrophobicity status is affected by morphology, age of culture, growth phase, and growth medium.

The surface status that appears "constitutive" is hydrophobic. When hydrophilic cells are treated with the appropriate reagents, the hydrophilic cells becomes hydrophobic (*e.g.* dithiothreitol treatment). Most *Candida* species are hydrophobic when grown at 37°C. So the question is not what makes a yeast cell hydrophobic, but what makes a *C. albicans* cell hydrophilic.

Hydrophilic and hydrophobic cell surface topography was evaluated by freeze-fracture analysis. A striking contrast was obtained (Hazen K C, and Hazen B W. (1992) *Infect Immun* 60, 1499-1508). The hydrophilic cells had surface fibrils that radiated and were relatively long (200 nm). The hydrophobic cells had a fibular layer but the fibrils were short (100 nm),

aggregated, blunt, and more randomly oriented. Other workers have suggested that the fibrillar layer of *C. albicans* is due to high molecular mass mannoproteins (Cassone A. (1989) *Curr Top Med Mycol* 3, 248-314; Hazen K C, and Glee P. M. (1994) *Can J Microbiol* 40, 266-272). Based on this, the possibility that the difference between hydrophobic and hydrophilic cells was the amount or type of protein mannosylation that was present at the cell surface was tested. No significant total cell wall protein or protein population differences between hydrophobic and hydrophilic cells were obtained (Masuoka J, and Hazen K C. (1997) *Microbiology* 143:3015-3021). However, differences in the mannose content of the mannoproteins were detected. Western blots with monoclonal antibodies raised against the outer mannosyl chain and the phosphomannosyl group indicated that hydrophobic cells have less phosphomannosyl groups (Masuoka J, and Hazen K C. (1999) *Glycobiology* 9:1281-1286). Thus, exposure of hydrophobic surface proteins depends on whether and which wall surface proteins are extensively glycosylated.

SUMMARY OF THE INVENTION

The present inventors have, for the first time, identified a number of cell wall proteins produced by yeasts that are hydrophobic and are involved in hydrophobically mediated pathogenic events. Antibodies to these proteins are effective inhibitors of hydrophobically mediated pathogenesis events.

More specifically, the present invention provides hydrophobic peptides from *C. albicans,* and antibodies to said peptides. The antibodies are directed against the hydrophobic proteins found on the yeast, and filamentous forms of these pathogenic yeasts. The antibodies recognize hydrophobic proteins on multiple pathogenic species of *Candida* and on other pathogenic yeasts and effectively block various pathogenic events that

involve the hydrophobic proteins. The antibodies are therefore unique in that they are directed against hydrophobic wall proteins and they are able to prevent pathogenic events. The antibodies offer a therapeutic advantage to other treatments for yeast diseases as they prevent a number of pathogenic events, most particularly attachment to host extracellular matrix, thereby preventing dissemination. The antibodies are also useful in diagnostic tests, for detecting the presence of *Candida*, and other pathogenic yeasts, in a biological sample.

The present invention is further directed to treating pathogenic yeast-related disease states. Methods for treating such diseases include the use of antibodies produced by the hybridoma cell lines F6-6C5-H4, F6-5F8-E10, and 5D8-A12CA and the use of peptides analogous in structure to the bonding domains exhibited on the surface of hydrophobic yeast cell wall proteins, to interfere with yeast cells' ability to bond to host tissues. More particularly, the peptides are of the general formula G-X_1-X_2-R, wherein G is glutamate or glutamine, X_1 is a bond or an amino acid, X_2 is an amino acid, and R is a tripeptide wherein at least one amino acid of the tripeptide is valine, leucine, isoleucine, phenylalanine, tyrosine, or tryptophan.

With the foregoing and other objects, advantages and features of the invention that will become hereinafter apparent, the nature of the invention may be more clearly understood by reference to the following detailed description of the preferred embodiments of the invention and to the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: Representative western blot of *Candida albicans* cell wall digests. Glucanase-extracted wall proteins from hydrophobic yeast cells (1), germ tube initials (2) and hydrophilic yeast cells (3) are shown. Proteins were visualized by their recognition by the polyclonal α-HICF6 antiserum or the

monoclonal antibodies (5F8, 6C5 and 5D8). Arrowheads in the α-HICF6 section indicate the positions of the 37, 38 and 40 kDa proteins.

Fig. 2: Inhibition of *C. albicans* adhesion to immobilized Fn (a, c) or Ln (b, d). Results of these experiments are presented in box plot form, in which the top and bottom of the box represent the 75th and 25th percentiles, respectively. The interior horizontal line indicates the median. The gray shaded area indicates the 95% confidence intervals about the median. These confidence intervals are calculated so that if shaded areas do not overlap, the medians are different at the 5% significance level (α=0.05). n=4, except for mIgG2a (n=6).

Fig. 3: Diagrammatic representation of the influence of amino acid sequence on surface exposure. A short sequence of consecutive hydrophobic amino acids surrounded by polar amino acids may not undergo conformational (inward) change (A) but a longer sequence does (B) unless it is constrained by anchors to other molecules (C).

Fig. 4: Influence of hydrophobic peptide conformation on interaction with hydrophobic sites on a target molecule. Two molecules may have hydrophobic regions at their molecular surfaces but interaction is constrained by structure.

Fig. 5: DNA sequence of 6C5 antigen and deduced amino acid sequence.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides three hydrophobic proteins derived from *Candida albicans* :6C5, 5D8, and 5F8. These cell wall surface proteins

contribute to the cell surface hydrophobicity of many yeast species, particularly that of *Candida albicans*. Cell surface hydrophobicity facilitates the adhesion of yeast cells to host tissue, an important initial step in pathogenic yeast cell colonization of host organisms. Prevention of this bonding to host tissue provides an effective means of treating yeast infections and conditions related to pathogenic yeast cell colonization.

The 6C5 protein antigen is one gene product of a family of four or five similar gene products in *C. albicans*. The gene sequence of 6C5 (Fig. 5) encodes a 337 amino acid protein with a predicted molecular weight of 38.228 kD and isoelectric point of 5.80, in close agreement with experimental determinations. The genetically tractable yeast *Saccharomyces cerevisiae* has a single homologous gene of unknown function that is very similar in protein sequence. The 6C5 monoclonal antibody epitope was mapped by phage display panning, revealing the following epitopes:

clone4: ~~~~~~RVDVGSEDAPSR~~
clone54: ~~~~SDRLEVGTEDLR~~~~
clone43: ~~~~~~DVEVGAEDALFS~~
clone13: ~IEARVEIDWSLD~~~~~~~
clone26: ~~GRNLEVDRALDT~~~~~~
clone_11: ~~RSMLELDVILEG~~~~~~
clone12: ~EGIGVEVEVVLE~~~~~~~
clone4-3-3: ~~~~~~EIDLKLEPGTRV~~
clone44: ~~~~RVELAHELEGVW~~~~
clone38: ~~~~~~ELD.AID.RPGIVV
clone39: ~~~VRLEID.QIDRGP~~~~
clone5-4-1: ~~~~SFEVD.PIDENSG~~~
clone1-8: GETQCVEIA.SID~~~~~~~

clone29: ~~IGFVERD.DIDAE~~~~~

clone57: ~~~~~~EID.SFELYLRER~

The protein sequence "EIDPID" can be found in the open reading frame at residue number 107. The protein sequence matching the monoclonal antibody epitope is not present in the other *C. albicans* sequences, nor is it present in the *S. cerevisiae* sequence.

The 5D8 antigen epitope has been mapped by phage display panning, and peptides showing 5D8 reactivity are aligned below. The putative consensus sequence for the epitope is "E(E/P/K)L(F or Y)(I or V)(S/T)." A *C. albicans* protein of approximately 40 kDa molecular weight with 5D8 immunoreactivity prepared by glass bead cell breakage has been analyzed by mass spectroscopy, and has been tentatively assigned as *C. albicans* actin. In a second mass spectroscopy analysis using material prepared by limited lyticase digestion, a protein immunoreactive with 5D8 shows no similarity with actin, and is currently undergoing further investigation.

Panned 5D8 antigen epitopes:

{clone 1} PLLPEPLFIELG

{clone 12} ~~~~EKLYISAWDHLN

{clone 19} ~RFVEPLYVTAAG

{clone 23} ~~~~EELFISRLSRAP

5D8 peptides derived by second mass spec. analysis:

PFVDAYPFNR

TDQTSNNNVA

TWSV(I/L)PT

The 5F8 antigen epitope has not been mapped by phage display. A *C.*

albicans protein extract generated by limited lyticase digestion containing a 40 kDa immunoreactive protein has been analyzed by mass spectroscopy. Peptides derived from the 40 kDa protein generated by digestion with trypsin show sequence similarity to 2 bacterial glucanases of higher molecular weight, one of 46 kDa apparent mass, and one of 58 kDa mass. Masses of some peptides show no similarity with these glucanases.

Mass-spec identified peptides:

IWIPR

CLDVR

WAYDAGSK

YQPQYGR

ALNPSHGIDVGK

LVQPAVQNDSDPNR

ANSALDGQGNLVITAR

DLQAPNDHVVGPIAR

GAAVQVWTCNGTGAQK

TTALTNPNQPDANSAGFYQDAR

Antibodies produced to the hydrophobic proteins of pathogenic yeast species including but not limited to *Candida* species are useful in therapies against the many types of infections and for diagnostics targeting those same proteins and microorganisms. Such infections include but are not limited to opportunistic and nosocomial. These antibodies may also be used as research and development tools (research tools). Both monoclonal and polyclonal antibodies are included as useful biological molecules and their individual components including the antigen binding region (F_{ab}) or the crystallization region (F_c) are useful in therapy diagnosis and research.

Specific peptides and derivatives isolated from the antibodies can be used to develop products of biotechnology as well. Biologically active molecules such as toxins, peptide drugs and enzymes can be attached to these antibodies in the development of novel therapies, diagnostics and research tools targeted toward yeast species. All of the above biological molecules can be targeted for human use or can be targeted for veterinary use. Such biological molecules are useful in preventative treatments of wounds, contaminated surfaces and surgical tools.

These peptides may also be used in the development of peptidomimetics and/or small organic molecules with therapeutic, diagnostic and/or research use.

The genes involved may be used in the development of aptamers, gene therapies, delivery systems for genes and/or in gene vector constructs for diseases related to yeast infection or as preventative therapies for patients at risk for such opportunistic and/or nosocomial infections.

In accordance with one embodiment of the present invention, antibodies are generated against hydrophobic yeast cell surface proteins. The antibodies are generated using standard techniques and specific antibodies are selected that bind to regions of the hydrophobic proteins that interfere with the yeast cells' ability to adhere to host tissue surfaces.

The antibodies of the present invention have been shown to recognize these binding regions of hydrophobic yeast cell wall proteins. The antibodies of the present invention include antibodies produced by the F6-6C5-H4, F6-5F8, and F6-5D8-A12 hybridoma cell lines, including polyclonal and monoclonal antibodies as well as any fragments of these antibodies exhibiting similar chemical activity. The F6-6C5-H4, F6-5F8-E10, and F6-5D8-A12 hybridoma cell lines have been deposited with the ATCC.

The proteins recognized by the antibodies of the present invention exhibit hydrophobic binding domains on the surface of yeast cell walls.

These binding domains are peptide sequences exhibited on the exterior surface of cell wall proteins. Antibodies directed to these antigens can inhibit yeast cell adhesion to host tissue by directly occupying these binding domains. Alternatively, yeast cell colonization may be blocked by occupying the sites on host tissue surfaces targeted by the yeast proteins. Introduction of peptides analogous in structure to the hydrophobic binding domains recognized by the present antibodies should effectively inhibit the binding of yeast cells to host tissue.

Antibodies according to the present invention can be raised against native or recombinant protein, or antigenic fragments thereof. The antibodies of the present invention may be prepared using known techniques. Monoclonal antibodies may be prepared using hydridoma technology as described by Kohler et al, *Nature* 256:495 (1975); Kohler et al, *Eur. J. Immunol.* 6:511 (1976); Kohler et al, *Eur. J. Immunol.* 6:292 (1976); Hammerling et al, in: *Monoclonal Antibodies and T-Cell Hybridomas*, Elsavier, NY, pages 563-681 (1981). Such antibodies produced by the methods of the invention are capable of protecting against yeast infection.

The term "antibody" includes both polyclonal and monoclonal antibodies, as well as fragments thereof, such as, for example, Fv, Fab and $F(ab)_2$ fragments which are capable of binding antigen or hapten. Such fragments are typically produced by proteolytic cleavage, such as papain, to produce Fab fragments or pepsin to produce $F(ab)_2$ fragments. Alternatively, hapten-binding fragments can be produced through the application of recombinant DNA technology or through synthetic chemistry.

As indicated, both polyclonal and monoclonal antibodies may be employed in accordance with the present invention. Of special interest to the present invention are antibodies which are produced in humans or are "humanized" (*i.e.*, non-immunogenic in a human) by recombinant or other technology. Humanized antibodies may be produced, for example, by

placing an immunogenic portion of an antibody with a corresponding, but non-immunogenic portion, chimeric antibodies. See, for example, Robinson et al, International Patent Publication PCT/US86/02269; Akira et al, European Patent Application 184,187; Taniguchi, M. European Patent Application 171,496; Morrison et al, European Patent Application 173,494; Neuberger et al, PCT Application WO86/01533; Cabilly et al, European Patent Application 125,023; Better et al, *Science* 240:1041-1043 (1988); Liu et al, *PNAS* 84:3439-3443 (1987); Liu et al, *J. Immunol.* 139:3521-3526 (1987); Sun et al, *PNAS* 84:214-218 (1987); Nishimura et al, *Cancer Research* 47:999-1005 (1987); Wood et al, *Nature* 314:446-449 (1985); and Shaw et al, *J. National Cancer Inst.* 80:1553-1559 (1988). General reviews of "humanized" chimeric antibodies are provided by Morrison, S.L. *Science* 229:1202-1207 (1985) and by Oi et al, *BioTechniques* 4:214 (1986).

The antibodies, or antibody fragments, of the present invention can be utilized to detect, diagnose, serotype, and treat yeast infection. In this manner, the antibodies or antibody fragments are particularly suited for use in immunoassays. Such immunoassays include diagnostic tests, for detecting the presence of *Candida*, and other pathogenic yeasts, in a biological sample.

Antibodies, or fragments thereof, may be labeled using any of a variety of labels and methods of labeling. Examples of types of labels which can be used in the present invention include, but are not limited to, enzyme labels, radioisotopic labels, non-radioactive isotopic labels, fluorescent labels, toxin labels, and chemiluminescent labels.

Examples of suitable enzyme labels include malate hydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast-alcohol dehydrogenase, alpha-glycerol phosphate dehydrogenase, triose phosphate isomerase, peroxidase, alkaline phosphatase, asparaginase, glucose

oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, acetylcholine esterase, etc.

Examples of suitable radioisotopic labels include ^{3}H, ^{125}I, ^{131}I, ^{32}P, ^{35}S, ^{14}C, ^{51}Cr, ^{57}To, ^{58}Co, ^{59}Fe, ^{75}Se, ^{152}Eu, ^{90}Y, ^{67}Cu, ^{217}Ci, ^{211}At, ^{212}Pb, ^{47}Sc, and ^{109}Pd.

Examples of suitable fluorescent labels include a ^{152}Eu label, a fluorescein label, an isothiocyanate label, a rhodamine label, a phycoerythrin label, a phycocyanin label, an allophycocyanin label, an o-phthaldehyde label, a fluorescamine label, etc.

Examples of suitable toxin labels include diphtheria toxin, ricin, and cholera toxin. Examples of chemiluminescent labels include a luminal label, an isoluminal label, an aromatic acridinium ester label, and imidazole label, and acridinium salt label, an oxalate ester label, a luciferin label, a luciferase label, an aequorin label, etc.

Those of ordinary skill in the art will know of other suitable labels which may be employed in accordance with the present invention. The binding of these labels to antibodies or fragments thereof can be accomplished using standard techniques commonly known to those of ordinary skill in the art. Typical techniques are described by, *inter alia,* Kennedy, J.H., et al (*Clin. Chim. Acta* 70:1-31 (1976)), and Schurs, A.H.W.M., et al, (*Clin. Chim. Acta* 81:1-40 (1977)). Coupling techniques mentioned in the latter are the glutaraldehyde method, the periodate method, the dimaleimide method, the m-maleimidobenzyl-N-hydroxy-succinimide ester method, all these methods incorporated by reference herein.

The detection of the antibodies (or fragments of antibodies) of the present invention may be improved through the use of carriers. Well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses

and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. Those skilled in the art will note many other suitable carriers for binding monoclonal antibody, or will be able to ascertain the same by use of routine experimentation.

By raising antibodies against hydrophobic yeast surface proteins which mimic the antigenicity of yeast, the antibodies raised against such recombinant proteins are neutralizing and protective antibodies. The antibodies are able to prevent subsequent infection of the same type of yeast from which the hydrophobic surface protein was derived. That is, if a recombinant or native hydrophobic surface protein from *C. albicans* is utilized to raise antibodies, these antibodies will protect against subsequent infection of *C. albicans*. Thus, the method of the present invention provides for the prevention, treatment or detection of infection by any yeast type.

The invention also provides for pharmaceutical compositions as the antibodies can also be utilized to treat yeast infections in a patient in need of such treatment. In the context of the present invention, a patient in need of treatment will be understood to be any mammal, particularly a human, infected with any yeast. The antibodies or monoclonal antibodies can be used in pharmaceutical compositions to target drug therapies to sites of yeast infection. In this manner, the drugs or compounds of interest are linked to the antibody to allow for targeting of the drugs or compounds. Methods are available for linking antibodies to drugs or compounds. See, for example, EP 0,146,050; EP 0,187,658; and U.S. Patent Nos. 4,673,573; 4,368,149; 4,671,958 and 4,545,988.

Such drug therapies include antibiotic agents, toxic agents and photoactivatable compounds, such as coumarin, psoralen, phthalocyanimes, methylene blue, eosin, tetracycline, chlorophylls, porphyrins and the like. Such groups can be attached to the antibodies by

appropriate linking groups. Antibody conjugates containing a photoactivatable compound are administered followed by irradiation of the target cells.

The antibody or antibody conjugates of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions such as by admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation are described, for example, in Remington's Pharmaceutical Sciences (16th Ed., Osol, A.Ed., Mack Easton PA (1980)). To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of antibody, either alone, or with a suitable amount of carrier vehicle.

The therapeutic or diagnostic compositions of the invention will be administered to an patient in therapeutically effective amounts. That is, in an amount sufficient to diagnose or treat yeast infection. The effective amount will vary according to the weight, sex, age and medical history of the patient. Other factors include, the severity of the patient's condition, the type of yeast, mode of administration, and the like. Generally, the compositions will be administered in dosages ranging from about 0.01 to about 2 mg/ml, more generally about 0.001 to about 20 mg/ml.

The pharmaceutically prepared compositions may be provided to a patient by any means known in the art including topical, oral, intranasal, subcutaneous, intramuscular, intravenous, intraarterial, parenteral, etc., with topical administration being particularly preferred.

Another aspect of the present invention involves the development of yeast type-specific vaccines. The vaccines of the invention are those that contain the necessary antigenic determinants to induce formation of neutralizing antibodies in the host; possess high immunogenic potential; are safe enough to be administered without danger of clinical infection; devoid

of toxic side-effects; suitable for administration by an effective route, for example, oral, intranasal, topical or parenteral; mimics the circumstances of natural infection; stable under conditions of long-term storage; and, compatible with the usual inert vaccine carriers.

The vaccines of the present invention include the conformationally correct recombinant yeast hydrophobic proteins or fragments thereof which provide the conformational epitopes present on the intact yeast. Such amino acid sequences of the yeast hydrophobic surface protein comprise the antigenic component of the vaccine. It may be necessary or preferable to covalently link the antigen to an immunogenic carrier, *i.e.,* bovine serum albumin or keyhole limpet hemocyanin. The vaccines of the invention may be administered to any patient susceptible to yeast infection. Human and non-animal mammals may benefit as hosts.

As noted above, administration of the vaccines may be by any route, including parenteral, but is preferably oral or intranasal, depending upon the natural route of infection. The dosage administered may be dependent upon the age, health, weight, kind of concurrent treatment, if any, and nature and type of the yeast. The vaccine may be employed in dosage form such as capsules, liquid solutions, suspensions, or elixirs, for oral administration, or sterile liquid formulations such as solutions or suspensions for parenteral or intranasal use. An inert, immunologically acceptable carrier is preferably used, such as saline or phosphate-buffered saline.

The vaccines will be administered in therapeutically effective amounts. That is, in amounts sufficient to produce a protective immunological response. Generally, the vaccines will be administered in dosages ranging from about 0.1 mg protein to about 20 mg protein, more generally about 0.01 mg to about 100 mg protein. A single or multiple dosages can be administered.

As more than one yeast type may be associated with yeast infections, the vaccines may comprise yeast hydrophobic surface proteins, or antigenic fragments thereof, from more than one type of yeast.

In accordance with one embodiment of this invention peptides, analogous in structure and activity to these hydrophobic binding domains, are provided. The introduction of these peptides provides for the inhibition of yeast cell binding to host tissue.

The peptides of the present invention are of the general formula:

$$G\text{-}X_1\text{-}X_2\text{-}R$$

wherein G is glutamate or glutamine; X_1 is a peptide bond or an amino acid; X_2 is an amino acid; and R is a sequence of three amino acids at least one of which is selected from the group: valine, leucine, isoleucine, phenylalanine, tyrosine, and tryptophan.

In accordance with this invention, X_1 and X_2 may be any natural or synthetic amino acid or any molecule capable of forming a peptide bond.

In one embodiment of this invention there is provided a peptide of the above formula, wherein G is Glutamate, X_1 is a peptide bond, X_2 is Proline, and R is selected from the group consisting of the tripeptide sequences:

Leucine-Tyrosine-Isoleucine;

Leucine-Tyrosine-Valine; and

Leucine-Phenylalanine-Isoleucine.

In another embodiment there is provided a peptide of the above formula, wherein G is Glutamate, X_1 is a peptide bond, X_2 is Proline, and R is the tripeptide sequence: Leucine-Phenylalanine-Valine.

The recognition of yeast cell surface proteins by the antibodies of the present invention enables a method of detecting the presence of pathogenic yeasts as well as other organisms exhibiting similar hydrophobic surface bonding domains. The antibodies of the present invention may be labeled by a number of detectable marker molecules known to those of ordinary skill

in the art. Labeled antibody may then be applied in a manner that brings the antibody into contact with the components of a target environment or target substance believed to contain antigens exhibiting these bonding domains. The respective amounts of labeled antibody bound to target molecules or labeled antibody left unbound may be used to determine the level of antigen presence. This method of detection may be performed *in vivo* or *in vitro*.

Further, this method of antigen detection may be performed by binding antibody to a solid support and bringing the target substance into contact with the bound antibody. Possible forms of solid support include a column of beads or a fixed surface of a container or vessel through which or within the target is brought into contact with the antibody. Antibody bound to a solid support need not be labeled as it will remain fixed to the solid support throughout the method.

In one embodiment of the present invention there is provided a method of antigen detection including the steps of passing the target suspected of infection through a column comprising one or a plurality of agarose beads with antibody bound to said beads, washing the column to remove any material not bound to the antibody with a substance with an affinity for the antibody insufficient to displace bound targets, and then passing a volume of a substance with a affinity sufficient to displace the bound target molecules to determine the amount of target antigen bound by the antibody.

In addition to detection, the recognition of hydrophobic binding domains by the antibodies of the present invention enables methods of treating yeast infection and related conditions. The hydrophobic cell wall proteins recognized by the antibodies of the present invention facilitate the binding of yeast cells to host tissue, an initial step in yeast cell pathogenesis. Thus, inhibition of this binding should prove to be an effective treatment of conditions associated with yeast infection.

This preliminary stage of infection may be inhibited by occupying the hydrophobic binding domains of the cell wall proteins or by occupying the host tissue sites to which these yeast cells bond. Such inhibition can be achieved through introduction of the antibodies or peptides of the present invention.

In one embodiment of this invention there is provided a method of treating a yeast infection and associated conditions wherein the antibodies of the present invention are applied topically to an area suspected of yeast infection in an amount sufficient to inhibit the binding of yeast cells to host tissue. Further, the antibodies may be applied within a pharmaceutically acceptable carrier capable of preventing antibody degradation prior to and during application. Many such carriers are well known in the art and include ointments, lotions, salves, and emulsions.

In another embodiment there is provided a method of treating a yeast infection and associated conditions wherein an amount of the peptides of the present invention is applied topically in an amount sufficient to inhibit the binding of yeast cells to host tissue. Further, the peptides may be applied within a pharmaceutically acceptable carrier capable of preventing the degradation of the peptides prior to and during application.

In another embodiment there is provided a method of treating a yeast infection and associated conditions wherein an amount of the peptides of the present invention sufficient to inhibit the binding of yeast cells to host tissue is introduced orally within a pharmaceutically acceptable carrier capable of preventing gastro-intestinal or other degradation of the peptides. Such carriers are well known in the art and include solid, liquid, and capsulated forms.

Pharmaceutical Compositions and Vaccines

Within certain aspects, polypeptides, polynucleotides, antibodies, T cells and/or binding agents described herein may be incorporated into pharmaceutical compositions or immunogenic compositions (*i.e.,* vaccines). Pharmaceutical compositions comprise one or more such compounds and a physiologically acceptable carrier. Vaccines may comprise one or more such compounds and an immunostimulant. An immunostimulant may be any substance that enhances or potentiates an immune response (antibody and/or cell-mediated) to an exogenous antigen. Examples of immunostimulants include adjuvants, biodegradable microspheres (*e.g.,* polylactic galactide) and liposomes (into which the compound is incorporated; *see e.g.,* Fullerton, U.S. Patent No. 4,235,877). Vaccine preparation is generally described in, for example, M.F. Powell and M.J. Newman, eds., "Vaccine Design (the subunit and adjuvant approach)," Plenum Press (NY, 1995). Pharmaceutical compositions and vaccines within the scope of the present invention may also contain other compounds, which may be biologically active or inactive. For example, one or more immunogenic portions of other antigens may be present, either incorporated into a fusion polypeptide or as a separate compound, within the composition or vaccine.

A pharmaceutical composition or vaccine may contain DNA encoding one or more of the polypeptides as described above, such that the polypeptide is generated *in situ*. As noted above, the DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacteria and viral expression systems. Numerous gene delivery techniques are well known in the art, such as those described by Rolland, *Crit. Rev. Therap. Drug Carrier Systems 15*:143-198, 1998, and references cited therein. Appropriate nucleic acid expression systems contain the necessary DNA sequences for

expression in the patient (such as a suitable promoter and terminating signal). Bacterial delivery systems involve the administration of a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an immunogenic portion of the polypeptide on its cell surface or secretes such an epitope. In a preferred embodiment, the DNA may be introduced using a viral expression system (*e.g.,* vaccinia or other pox virus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic (defective), replication competent virus. Suitable systems are disclosed, for example, in Fisher-Hoch et al., *Proc. Natl. Acad. Sci. USA 86*:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; Flexner et al., *Vaccine 8*:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973; U.S. Patent No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91/02805; Berkner, *Biotechniques 6*:616-627, 1988; Rosenfeld et al., *Science 252*:431-434, 1991; Kolls et al., *Proc. Natl. Acad. Sci. USA 91*:215-219, 1994; Kass-Eisler et al., *Proc. Natl. Acad. Sci. USA 90*:11498-11502, 1993; Guzman et al., *Circulation 88*:2838-2848, 1993; and Guzman et al., *Cir. Res. 73*:1202-1207, 1993. Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in Ulmer et al., *Science 259*:1745-1749, 1993 and reviewed by Cohen, *Science 259*:1691-1692, 1993. The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells. It will be apparent that a vaccine may comprise both a polynucleotide and a polypeptide component. Such vaccines may provide for an enhanced immune response.

It will be apparent that a vaccine may contain pharmaceutically acceptable salts of the polynucleotides and polypeptides provided herein. Such salts may be prepared from pharmaceutically acceptable non-toxic bases, including organic bases (*e.g.,* salts of primary, secondary and tertiary

amines and basic amino acids) and inorganic bases (*e.g.*, sodium, potassium, lithium, ammonium, calcium and magnesium salts).

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration. Compositions of the present invention may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (*e.g.*, polylactate polyglycolate) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268; 5,075,109; 5,928,647; 5,811,128; 5,820,883; 5,853,763; 5,814,344 and 5,942,252.

Such compositions may also comprise buffers (*e.g.*, neutral buffered saline or phosphate buffered saline), carbohydrates (*e.g.*, glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents such as EDTA or glutathione, adjuvants (*e.g.*, aluminum hydroxide), solutes that render the formulation isotonic, hypotonic or weakly hypertonic with the blood of a recipient, suspending agents, thickening agents and/or preservatives. Alternatively, compositions of the present invention may be formulated as a lyophilizate. Compounds may also be encapsulated within liposomes using well known technology.

Any of a variety of immunostimulants may be employed in the vaccines of this invention. For example, an adjuvant may be included. Most adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bortadella pertussis* or *Mycobacterium tuberculosis* derived proteins. Suitable adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); AS-2 (SmithKline Beecham, Philadelphia, PA); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF or interleukin-2, -7, or -12, may also be used as adjuvants.

Preferred adjuvants for use in eliciting a predominantly antibody response include, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL), together with an aluminum salt. MPL adjuvants are available from Corixa Corporation (Seattle, WA; *see* US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 96/02555 and WO 99/33488. Immunostimulatory DNA sequences are also described, for example, by Sato et al., *Science* 273:352, 1996. Another preferred adjuvant is a saponin, preferably QS21 (Aquila Biopharmaceuticals Inc., Framingham, MA), which may be used alone or in combination with other adjuvants. For example, an enhanced system involves the combination of a monophosphoryl lipid A and saponin

derivative, such as the combination of QS21 and 3D-MPL as described in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol, as described in WO 96/33739. Other preferred formulations comprise an oil-in-water emulsion and tocopherol. A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil-in-water emulsion is described in WO 95/17210.

Other preferred adjuvants include Montanide ISA 720 (Seppic, France), SAF (Chiron, California, United States), ISCOMS (CSL), MF-59 (Chiron), the SBAS series of adjuvants (*e.g.,* SBAS-2 or SBAS-4, available from SmithKline Beecham, Rixensart, Belgium), Detox (Corixa, Seattle, Washington), RC-529 (Corixa, Seattle, Washington) and Aminoalkyl glucosaminide 4-phosphates (AGPs).

Any vaccine provided herein may be prepared using well known methods that result in a combination of antigen, immune response enhancer and a suitable carrier or excipient. The compositions described herein may be administered as part of a sustained release formulation (*i.e.,* a formulation such as a capsule, sponge or gel (composed of polysaccharides, for example) that effects a slow release of compound following administration). Such formulations may generally be prepared using well known technology (*see, e.g* ., Coombes et al., *Vaccine 14*:1429-1438, 1996) and administered by, for example, oral, rectal or subcutaneous implantation, or by implantation at the desired target site. Sustained-release formulations may contain a polypeptide, polynucleotide or antibody dispersed in a carrier matrix and/or contained within a reservoir surrounded by a rate controlling membrane.

Carriers for use within such formulations are biocompatible, and may also be biodegradable; preferably the formulation provides a relatively constant level of active component release. Such carriers include microparticles of poly(lactide-co-glycolide), as well as polyacrylate, latex,

starch, cellulose and dextran. Other delayed-release carriers include supramolecular biovectors, which comprise a non-liquid hydrophilic core (*e.g.*, a cross-linked polysaccharide or oligosaccharide) and, optionally, an external layer comprising an amphiphilic compound, such as a phospholipid (*see e.g.*, U.S. Patent No. 5,151,254 and PCT applications WO 94/20078, WO/94/23701 and WO 96/06638). The amount of active compound contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

Vaccines and pharmaceutical compositions may be presented in unit-dose or multi-dose containers, such as sealed ampoules or vials. Such containers are preferably hermetically sealed to preserve sterility of the formulation until use. In general, formulations may be stored as suspensions, solutions or emulsions in oily or aqueous vehicles. Alternatively, a vaccine or pharmaceutical composition may be stored in a freeze-dried condition requiring only the addition of a sterile liquid carrier immediately prior to use.

In another embodiment there is provided a method of treating a yeast infection and associated conditions wherein an amount of the peptides of the present invention sufficient to inhibit the binding of yeast cells to host tissue is introduced intravenously within a pharmaceutically acceptable carrier capable of preventing the degradation of the peptides. Further, said peptides and carrier may be introduced intraparietally or intramuscularly. In addition to a pharmaceutically acceptable carrier, any method of treatment may further be augmented by the addition of an adjuvant to initiate immune system activity. Many such adjuvants are well known in the art.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE 1

Adhesion of microorganisms to host tissue is an initial step in the colonization of new environments. For certain organisms, such as the opportunistic fungal pathogen *Candida albicans,* adhesion to multiple host substrata contributes to dissemination of disease. Cell surface hydrophobicity (CSH) plays an important role in the adhesive properties of *C albicans* cells. Hydrophobic cells bind more readily than hydrophilic cells to epithelial cells and plastics, and display more generalized binding to host tissues Furthermore, hydrophobic cells demonstrate greater resistance than hydrophilic cells to phagocytic killing, and are more virulent in mice. Thus, CSH is an important factor in the development of disease caused by *C albicans.*

Cell wall proteins are thought to contribute to, if not determine, the CSH status of *C albicans* cells. Hydrophobic proteins have been identified and extracted from the *C. albicans* cell wall. Previous work in our laboratory demonstrated that these proteins are expressed on the surface of *C. albicans* cells *in vivo.* Structural and functional evaluation of individual hydrophobic cell wall proteins would thus provide insight into the specific role of CSH in host-fungal interactions.

This evaluation was begun by comparing ^{125}I surface labeled wall proteins from hydrophilic and hydrophobic cells. This comparison identified a 38 kDa protein which appeared to be unique to hydrophobic cells and which fell into the molecular mass range (30-40 kDa) of the hydrophobic cell wall proteins retained by Hydrophobic Interaction Chromatography (HIC)-HPLC. Preliminary characterization of several of the hydrophobic

proteins indicated that they may be glycosylated since they bound the lectin Concanavalin A in Western blots.

Whether these proteins served function relevant to pathogenesis in addition to contributing to the hydrophobic character of the cell was not clear. Several groups, using affinity chromatography and western blotting techniques, have recently identified cell wall proteins that bind to extracellular matrix (ECM) proteins. The ECM proteins form in host tissue a substratum which may provide binding sites for yeast adhesion. The identified ECM-binding proteins of *C. albicans* have intermediate molecular masses (30-70 kDa), similar to the hydrophobic proteins

Recent work in our laboratory showed a relationship between CSH and attachment of *Candida* cells to ECM proteins. In particular, our lab demonstrated that proteins in the 30-70 kDa size range bind both fibronectin and laminin. Thus, hydrophobic proteins could contribute to pathogenesis by mediating attachment to host ECM proteins which are located throughout the vascular walls and interstitial sites.

We hypothesized that distinct exposed surfaces on the hydrophobic proteins are responsible for CSH status, and that these proteins are involved, at least in part, in the adhesion of *Candida* cells to host tissue. The role of these proteins in adhesion involves their interaction with the ECM proteins, and this interaction between the hydrophobic proteins and the ECM proteins could involve the hydrophobic regions on both sets of proteins. As part of our ongoing investigation of this hypothesis, we present here analyses of three monoclonal antibodies that recognize hydrophobic cell wall proteins (37, 38 and 40 kDa). These antibodies were selected for further study because of the differential distribution of their antigens among various pathogenic *Candida* species. Initial characterization of the three monoclonal antibodies and their protein antigens and assessment of the antigenic distribution in the *Candida* cell wall are described.

MATERIALS AND METHODS

Protein reagents

Human fibronectin (hFn) was obtained from Promega. Mouse laminin (mLn, ultrapure) was purchased from Collaborative Biomedical Products (Becton Dickinson). Mouse IgG2a mouse IgM, and alkaline phosphatase (AP)-conjugated goat α-mouse IgG were purchased from Sigma Chemical Co. AP-conjugated goat α-mouse was obtained from Jackson ImmunoResearch Laboratories.

Strains and culture conditions

C. albicans isolates were cultured as previously described [Hazen, K.C. and Hazen, B.W. (1993) *FEMS. Microbiol. Lett.* 107, 83-88; Hazen, K.C. and Hazen, B.W. (1987) *J. Microbiol. Methods.* 6, 289-299]. Briefly, cells were grown to stationary phase in phosphate-buffered (pH 7.2) yeast nitrogen base (plus amino acids, Difco Laboratories, Detroit, MI) containing 2% (w/v) glucose (YNB2G). Cells grown to stationary phase at 23°C were hydrophobic (Cell Surface Hydrophobility (CSH ≥ 95%); those grown to stationary phase at 37°C were hydrophilic (CSH ≤ 5%).

When germinated *C. albicans* cell were required, stationary phase yeast cells from 23°C cultures were incubated (2 h, 37°C, 1.0×10^6 cells ml^{-1}) in Auto-Pow minimal essential medium with Earle's salts, pH 6.8 (Flow Laboratories) supplemented with biotin (250 mg l^{-1}), glucose (9 g l^{-1}), glycine (1 g l^{-1}) and HEPES (6g l^{-1}) as previously described (Hazen, K.C. and Hazen, B.W. (1987) *Microbiol. Immunol* 31, 497-508). Only hydrophobic stationary phase yeast cells were induced to germinate because germination of hydrophilic yeasts produces pseudohyphae rather than true germ tubes.

Cell wall preparations (see below) from seven species of *Candida* were compared for similarities in hydrophobic protein complement. *C.*

albicans LGH1095 (the strain used for protein characterization studies) and LGH870 have already been described (Antley, P.P. and Hazen, K.C. (1988) *Infect Immun.* 56, 2884-2890). *Candida kefyr, C. krusei, C. parapsilosis,* and *C. tropicalis* isolates were clinical specimens isolated in the University of Virginia Medical Center Clinical Microbiology Laboratory. *Candida glabrata* ZB5 was taken from our frozen stock collection. Other isolates were obtained from the American Type Culture Collection (ATCC). Isolates were subcultured three times at 37°C in YNB2G.

Release and isolation of cell wall proteins

Proteins were released from the walls of stationary phase yeast or germinated cells by the previously described rapid crude digest (RCD) procedure (Glee, P.M. et al. (1995) *Infect Immun.* 63: 1373-1379; Hazen, K.C. and Hazen, B.W. (1992) *Infect Immun.* 60, 1499-1508). Digestion was stopped by centrifugation (2 × 10 min. at 14,000 g), followed by removal of the supernatant fluid, when the concentration of released protein reached ~300-500 mg ml^{-1}, (Coomassie Plus assay, Pierce Chemical Co.). Protease inhibitors were replenished in the final supernatant fluid and the final protein concentration was determined (bicinchoninic acid protein assay, Pierce) (Smith, P.K.,(1985) *Anal. Biochem.* 150, 76-85). This method of digestion minimizes cytoplasmic contaminants based on the lack of ghost cells present at the end of the digestion period. The RCD material was not dialyzed, as previous work from our laboratory showed that the majority of wall protein is lost during dialysis (Glee, P.M., et al.(1996) *J. Med. Vet. Mycol.* 34, 57-61).

Polyclonal and monoclonal antibody production

Separation of the RCD proteins by hydrophobic interaction chromatography (HIC)-HPLC and the production of a polyclonal antiserum

(designated α-HICF6 pAb) against the hydrophobic HIC-HPLC fractions (6 and 7, HICF6/7) has been previously described (Glee, P.M., et al.(1995) *Infect Immun.* 63, 1373-1379; Hazen, K.C. and Hazen, B.W. (1992) *Infect Immun.* 60, 1499-1508). Monoclonal antibodies (mAb) to *C. albicans* hydrophobic proteins were produced in collaboration with the University of Virginia Medical Center Hybridoma Facility (Chang, J.H., et al.(1994) *Methods. Enzymol.* 254, 430-435). A/J or BALB/c mice (maintained in an American Association for the Accreditation of Laboratory Animal Care approved facility) were immunized with hydrophobic proteins from HICF6 as previously described (Glee, P.M., et al.(1995) *Infect Immun.* 63, 1373-1379). Antiserum reactivity was monitored by western blot analysis of HICF6/7 proteins. A final intrasplenic boost of approximately 3 µg of gel-purified (Glee, P.M., et al.(1996) *J. Med. Vet. Mycol.* 34, 57-61) 32-40 kDa proteins from HIC-HPLC fraction 6 was administered (Spitz, M., et al.(1984) *J. Immunol Methods.* 70, 3943). Splenocytes were fused with Sp/O myeloma cells, plated in 96-well microliter plates, and the supernatant fluids screened for antibody ELISA. Positive wells were screened by western blot analysis of HICF6/7 proteins. Reactive hybridomas were subcloned and retested by western blot. Monoclonal antibodies were evaluated for isotype (Pharmingen). The three hybridomas chosen for further study were designated F6-6C5-H4 (6C5, derived from A/J mice), F6-5D8-A12 (5D8, derived from BALB/c mice) and F6-5F8-E10 (SF8, derived from BALB/c mice). Ascitic fluid was produced in BALB/c mice.

Electrophoresis and western blotting

Cell wall proteins from RCD were separated by SDS-PAGE using 12.5% (w/v) acrylamide resolving gels. Cell wall proteins were also separated by preparative isoelectric focusing (Rotofor, Bio-Rad). Ampholytes (pH 4-7, Bio-Lytes, Bio-Rad) were mixed with the RCD solution

to a final concentration of 2% (w/v). The solution was introduced into the focusing chamber and electrophoresed according to the manufacturer's specifications. The fractions were harvested following focusing and loaded onto a SDS-PAGE slab gel as above. Following electrophoresis, the separated proteins were-transferred to nitrocellulose (BA-85, Schleicher and Schuell) membranes as described (Glee, P.M., et al.(1995) *Infect Immun.* 63, 1373-1379).

Strips were cut from the membranes, rehydrated with water and soaked in Dulbecco's phosphate buffered saline, pH 7.2 (DPBS). Strips were blocked by incubation (37 °C, 1 h) in DPBS containing 5% (w/v) dry nonfat milk and 0.2% (v/v) Tween. This solution was used for all antibody dilutions and wash steps. Blocked strips were incubated (37 °C, 1-2 h) in primary antibody solution. 6C5 was used at a 1:2000 dilution of ascites. 5F8 and 5D8 were used as the hybridoma culture supernatant fluid without dilution. The polyclonal antiserum, α-HICF6 pAb, was used at a 1:1000 dilution. Strips were washed three-times (10 min each) and-incubated (37°C, 1 h) in secondary antibody solution. Secondary antibodies were alkaline phosphatase (AP)conjugated goat α-mouse IgG (1:500) to recognize 6C5 or 5D8, and AP-conjugated goat α-mouse IgM (1:500) for 5F8. Final washes and detection were as described (Glee, P.M., et al.(1995) *Infect Immun.* 63, 1373-1379). A mixture of the two secondary antibodies was used for-lanes treated with the polyclonal antiserum.

Cell adhesion assay

Cell adhesion assays were carried out in 48-well flat-bottom, non-tissue culture treated, polystyrene multiwell plates (Falcon #1178). Fn and Ln were diluted to 66.7 μg ml^{-1} in DPBS. The distilled water and all buffer solutions used in this assay were sterilized by autoclaving. The wells were

incubated with 150 µl of the diluted ECM protein solution (10 µg protein) overnight at 4°C. The wells were then washed three times with cold DPBS.

The wells were blocked with 250 µl of DPBS containing immunoglobulin. The immunoglobulin used for blocking was purified from normal mice, used at the same concentration as the test antibody, and of the same isotype as the-test antibody. The wells were incubated in blocking solution for 2 h at room temperature. During the block incubation, cells from a third transfer (23 °C) culture were harvested by centrifugation and washed twice with cold distilled water. Germination was induced at this point, after which the germ tube initials were harvested and washed as above. Cell concentration, CSH and percent germination were determined. From the washed cell suspension, 3×10^6 cells were transferred to each of several glass tubes containing 2 ml of treatment solution.

For treatments using 6C5 and 5D8, cells were added to a solution of ascites diluted (1:300 and 1:500, respectively) in DPBS. The final concentration of antibody was 12 µg mrl. For experiments using 5F8, cells were added to a solution of ascites diluted 1:300 in DPBS (final concentration 23 µg ml^{-1}). Irrelevant mouse IgG2a and IgM were used as controls for 6C5/5D8 and 5F8, respectively, at the same. concentration. Cells were incubated in the antibody solution at room temperature for 15 minutes, centrifuged and washed (once) with DPBS. Following treatment, a final cell count was performed and the cell concentration adjusted to 8×10^2 cells ml^{-1}, if necessary.

Immediately before the cells were added to the wells, the blocking solution was removed and the wells gently washed once with DPBS. Following the wash, 200 cells from each treatment were added to their respective wells. The plates were then incubated at 37 °C for 15 min. The wells were gently washed three times with DPBS and covered with 300 µl of 1.7% (w/v) molten corn meal agar (CMA, Difco) previously tempered at 45

°C. The agar was allowed to solidify and the plates were incubated overnight at 37 °C. A replica aliquot of 200 cells from each treatment was used to inoculate a CMA plate. These plates were also incubated overnight at 37 °C. Percent adhesion was calculated by: (colonies per well / colonies per CMA plate) × 100. All samples were done in duplicate.

Adhesion data were analyzed in terms of percent relative binding. Binding in the presence of test or control antibody was compared to binding in the absence of antibody, which was defined as I 00%. The final data were visualized for comparison using a commercially available statistics package (Data Desk, Data Description, Inc.).

Two aspects of the cell adhesion experimental design are significant. First, the ECM proteins are used in a polymerized, immobilized state, as would be found in the ECM of the host/patient. Second, yeast cells are induced to germinate because the proteins, recognized by the three mAb are exposed or expressed predominantly on germ tubes.

RESULTS

Characterization of the monoclonal antibodies

Results of isotyping indicated that 6C5 and 5D8 are IgG2a and 5F8 is IgM. All three antibodies are κ haplotype. Western blots of RCD proteins from hydrophobic cells were probed with the three mAb. 6C5, 5F8 and 5D8 recognize proteins with an approximate molecular mass of 38, 40 and 37 kDa, respectively (Fig. 1, lane 1). Separation of RCD proteins based on isoelectric point (Rotofor, Bio-Rad) and subsequent western blot analysis indicated that the 37, 38 and 40 kDa proteins have an approximate pI of 6.2, 7.3, and 6.4, respectively. Proteins of these molecular masses were also among the RCD proteins recognized by a polyclonal serum generated against the hydrophobic wall proteins (Fig. 1). The mAb were used to probe blots of HIC-HPLC fractions. Results showed that the antigens for the mAb

were present only in HIC-HPLC fractions 5-7, with the greatest. concentration in fraction 6. The latter results indicate that the proteins recognized by 6C5, 5D8, and 5F8 are in the hydrophobic wall protein set.

Distribution of proteins recognized by the mAb

RCDs of hydrophilic yeast cells and germ tubes were compared with hydrophobic cells (Fig. 1). The three monoclonal antibodies recognized similar proteins from all three cell types. 5D8 recognized additional proteins in gerin tubes (Fig. 1, lane 2) and hydrophilic cells (Fig. 1, lane 3). It is unknown if the 38 kDa protein recognized by 6C5, present in cell walls from both hydrophobic and hydrophilic cells, is the same protein identified by ^{125}I surface labeling, which was detected only in hydrophobic cells (see above). One possibility is that more extensive, or altered, cell wall protein glycosylation may make iodination sites unavailable in hydrophilic cells.

The monoclonal antibodies were used to probe blots of wall proteins extracted from several species of *Candida*. As already seen by western blots and IFA, the wall of *C. albicans* contains all three proteins. *Candida tropicalis* cell wall extracts contained proteins which reacted with 6C5 and 5D8, however the 6C5-reactive protein was 54 kDa rather than 38 kDa. *C. kefyr* cell wall preparations contained proteins which bound 5F8 and 6C5. In these extracts, 5F8 also recognized a 59 kDa and a 55 kDa protein. The protein recognized by 6C5 was 36 kDa rather than 38 kDa. None of the three antibodies recognized proteins from lyticase digests of cell walls from *C. glabrata, C. guilliermondii, C. parapsilosis,* or *C. krusei.* A possibility is that it is the epitope, rather than the protein *per se,* which is not present in the other *Candida* species. Another possibility is that differences in cell wall construction exist among the various species. Preliminary results have indicated that the 40 kDa protein (though not the 38 or 37 kDa protein) is present in all the *Candida* species examined, but that additional extraction

methods are required for its release. This differential expression among the various *Candida* species, although potentially limited to epitopes, reinforced our interest in these three proteins and our efforts towards their characterization.

Antibody inhibition of cell binding to ECM

The ability of the mAb to block binding of intact cells to fibronectin (Fn) and Laninin (Ln) was investigated. In several separate experiments, the datasets included outliers. The outlier points could not be attributed to any systematic or investigator error. Because it is less influenced by extreme values,

the median was chosen as the more appropriate comparative parameter.

Pretreatment of germ tube initials with the antibodies significantly decreased the observed binding of cells to immobilized ECM proteins relative to untreated cells, shown as a decrease in relative binding (Fig. 2). Pretreatment of cells with irrelevant antibody or control medium also resulted in a decrease of cell attachment possibly due to steric effects from protein nonspecifically adsorbed to the cell surface.

Although 5F8 significantly reduced cell adhesion to Ln relative to control (Fig. 2d), this was not the case for cell adhesion to Fn (Fig. 2c). The decrease in cell binding to both Fn and Ln due to pretreatment of cells with either 6C5 or 5D8 was greater than that due to control (Fig. 2a & 2b). From these results, we conclude that the proteins recognized by the Mab are ECM binding proteins. Furthermore, the cell adhesion results relative to the irrelevant antibody controls also indicate that- the interaction of the 38 and 40 kDa proteins with Fn and Ln has some degree of specificity.

DISCUSSION

HPLC-fractionated cell wall proteins were used to generate three monoclonal antibodies, each of which recognizes a different hydrophobic protein. Using these monoclonal antibodies, we determined that the three antigens are in the cell wall and each monoclonal antibody was able to inhibit whole cell attachment to immobilized ECM. Thus, the results suggest that hydrophobic cells may adhere to host tissues via a contribution of hydrophobic cell wall protein attachment to ECM. If correct, then *C. albicans* possesses multiple surface molecules capable of ECM recognition.

Binding to the extracellular matrix of proteins on the cell surface of microorganisms is considered to play an important role in adhesion (for reviews *see* Roberts, D.D. (1990) Am. *J. Respir. Cell Mol. Biol.* 3, 181-186; Calderone, R.A..(1993) *Trends. Microbiol.* 1, 55-58; Patti, J.M. and Höök, M. (1994) *Curr. Op. Cell. Biol.* 6, 752-758; Pendrak, M.L. and Klotz, S.A. (1995) *FEMS. Microbiol. Lett* 129, 103-114). *C. albicans* cells have been shown to bind to several ECM proteins *in vitro* and *in vivo,* including fibronectin (Skerl, K.G., et al.(1984) *Can. J. Microbiol.* 30, 221-227; Scheld, W.M., et al. (1985) *Proc. Soc. Exp. Biol. Med.* 180, 474-482; Jakab, E., et al.(1993) *Apmis.*101, 187-193), laminin (Lopez-Ribot, J.L., et al.(1994) *Infect Immun.* 62,742-746; Bouchara, J.-P. (1990) *Infect Immun.* 58, 48-54), fibrinogen (Bouali, A., et al. (1986) *J. Med. Vet. Mycol.* 24, 345-348; Bendel, C.M., et al.(1993) *J. Clin. Invest.* 92, 1840-1849; Robert, R., et al. (1991) *FEMS. Microbiol. Lett* 78, 301-304), fibrin (Maisch, P.A. and Calderone, R.A. (1980) *Infect. Immun.* 27, 650-656), and entactin (Lopez-Ribot, J.L. and Chaffin, W.L. (1994) *Infect Immun.* 62, 4564-4571). Gale et al. ((1998) *Science.* 279, 1355-1358) have cloned a gene, *INT1*, which encodes an integrin-like protein and was subsequently linked to hyphal growth, virulence and adhesion. The cited studies have generally reported identification of individual ECM binding proteins. Comparison of these reports often reveals

that several proteins of differing molecular masses can each bind the various ECM proteins. For example, *C. albicans* wall proteins of 37, 60, 62, 67, and 68 kDa have been observed to bind laminin (Lopez-Ribot, J.L., et al.(1994) *Infect Immun.* 62,742-746; Bouchara, J.-P. (1990) *Infect Immun.* 58, 48-54). These molecular masses are very similar to those of proteins reported to bind fibrinogen (Casanova, M., et al. (1992) *Infect Immun.* 60,4221-4229), fibronectin (Klotz, S.A. et al. (1994) *Infect Immun.* 62,4679-4681), C3d (Calderone, R.A., et al.(1988) *Infect. Immun.* 56, 252-258; Saxena, A. and Calderone,.R.A. (1990) *Infect Immun.* 58, 309-314) and iC3b (Kanbe, T., et al. (1991) *Infect Immun.* 59, 1832-1838). Although there is some evidence that this multiplicity is due to protein glycosylation (Kanbe, T., et al. (1991) *Infect Immun.* 59, 1832-1838), it has not been demonstrated that these proteins are structurally related to each other.

The monoclonal antibodies described here recognize proteins that were first characterized based on their hydrophobicity rather than, as has been done most often, by direct functional assays. The common aspect of hydrophobicity may provide evidence for the structural relatedness mentioned above. We hypothesize that the fungal cell wall proteins and the ECM proteins interact through their hydrophobic regions.

The peptide sequence RGD has been accepted as the cell binding site on ECM proteins (Ayad, S., et al. (1994) *The Extracellular Matrix FactsBook.* Academic Press,. Inc.,San. Diego, CA, 1-163). However, several groups have reported that RGD alone is not sufficient to significantly inhibit *C. albicans* binding to ECM and host cells (Bendel, C.M., et al.(1993) *J. Clin. Invest.* 92, 1840-1849; Negre, E., et al. (1994) *J. Biol. Chem.* 269,22039-22045; Ollert, M.W., et al. (1993) *Infect. Immun.* 61, 4560-4568; Klotz, S.A. and Smith, R.L. (1991) *J. Infect. Dis.* 163,-604-610). The results presented here suggest that, beyond the RGD sequence,

hydrophobicity is also a shared trait and may contribute to a common mechanism driving, ECM-cell interactions.

The involvement of cell surface hydrophobicity in adhesion extends to other microorganisms as well. Vázquez-Juárez, et al. ((1997) *Mol. Mar. Biol. Biotechnol.* 6, 64-71) reported the involvement of CSH in the attachment of yeast strains to trout intestine. Davies, et al. ((1996) *Parasitology.* 112, 553-559) showed that there is a hydrophobic interaction between the bacterium *Pasteuria penetrans* and its host nematode. Courtney, et al. ((1990) in: *Microbial Cell Surface Hydrophobicity* (Doyle, R.J. and Rosenberg, M., Eds.), American Society for Microbiology, Washington, D.C. pp. 361-386) showed that fibronectin interacts hydrophobically with the *Streptococcus* cell surface.

While the invention has been described and illustrated herein by references to various specific material, procedures and examples, it is understood that the invention is not restricted to the particular material, combinations of material, and procedures selected for that purpose. Numerous variations of such details can be implied and will be appreciated by those skilled in the art.